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Biochimica et Biophysica Acta 1452 (1999) 197–208

BIOCHIMICA ET BIOPHYSICA ACTA

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A role for phosphatidylinositol 3-kinase in platelet aggregation in response to low, but not high, concentrations of PAF or thrombin

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Received 10 June 1999; received in revised form 2 September 1999; accepted 9 September 1999

Abstract

In this study we show that platelet activating factor (PAF) activates PI 3-kinase over a rapid time course that correlates closely with the aggregation response. Tyrosine kinases are involved in this response, since there is increased PI 3-kinase activity associated with tyrosine-phosphorylated proteins. PI 3-kinase inhibitors were used to probe the dependence of PAF-induced aggregation on PI 3-kinase. Both wortmannin and LY-294002 inhibited PAF-induced aggregation that correlated with PI 3-kinase inhibition only when using lower concentrations of PAF giving reversible aggregation (primary phase). Similar results were obtained with human platelets using thrombin or thrombin receptor activating peptide. The same pattern of response was observed when activation of GPIIbIIIa was assessed by flow cytometry, i.e., PI 3-kinase inhibitors blocked integrin activation only when lower concentrations of agonist were used. We suggest that PI 3-kinase is important for reversible (primary) aggregation of platelets in response to PAF or thrombin, perhaps by contributing to the 'inside-out' activation of the platelet integrin GPIIbIIIa, only when submaximal concentrations of agonists are used. The lack of effect of PI 3-kinase inhibitors, when high concentrations of agonist are used, suggests that PI 3-kinase-independent pathways contribute to aggregation under these conditions.

Keywords: Platelet; Signal transduction; Phosphatidylinositol 3-kinase; Enzyme inhibition

1. Introduction

Numerous studies have examined signaling pathways leading to activation of platelets in response to various agonists. Despite the fact that platelets are cell fragments that lack a nucleus, they maintain many of the signaling pathways seen in nucleated growing cells. For example, it has been shown that platelets contain a very high concentration of several tyrosine kinases, particularly non-receptor kinases such as *src* [1,2]. Furthermore, platelet agonists such as thrombin or platelet activating factor cause an increase in tyrosine phosphorylation that has been shown to be important for platelet function [3–5]. However, it may be that some of the enzymes are

Abbreviations: LY-294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; PAF, platelet-activating factor; PI, phosphatidylinositol; PI-4-P, phosphatidylinositol 4-phosphate; PI-3,4-P_2, phosphatidylinositol 3,4-bisphosphate; PI-4,5-P_2, phosphatidylinositol 4,5-bisphosphate; PIP_3, phosphatidylinositol(3,4,5)-trisphosphate; TLC, thin-layer chromatography; TRAP, thrombin receptor activating peptide; WM, wortmannin

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0167-4889/99/$ – see front matter
PII: S0167-4889(99)00125-1

activated only as remnants from platelet precursor cells, megakaryocytes, and they may not carry out any functional role in platelet activation.

A key signaling enzyme that is generally associated with cell growth and survival is PI 3-kinase. PI 3-kinase may also fulfill other functions not necessarily involved in cell growth, including effects on cytoskeleton that may be mediated via the small GTP-binding protein, rac [6–9]. PI 3-kinase has been shown to be activated in platelets in response to thrombin [10,11]. While the exact role of PI 3-kinase in platelets is not yet known, several studies have investigated its involvement in aggregation; one study suggested that the activation of PI 3-kinase in platelets stimulated with a thrombin receptor agonist peptide (TRAP) is important only for irreversible platelet aggregation, but not for primary aggregation [12].

In this study we show that platelet activating factor, an alkylphospholipid with potent proaggregatory properties [13] as well as a stimulator of leukocyte function [14], causes activation of PI 3-kinase in rabbit platelets, following a time course that correlates with aggregation. We find that inhibition of PI 3-kinase using wortmannin (WM) or LY-294002 could completely inhibit aggregation induced by low concentrations of PAF, while irreversible aggregation induced by higher concentrations of PAF was not inhibited to the same extent. Similarly, in human platelets, PI 3-kinase inhibitors could inhibit aggregation only when suboptimal concentrations of thrombin were used. These results support a model in which PI 3-kinase-dependent events play an essential role in platelet aggregation when low concentrations of agonists are used. At high agonist concentrations, there is no need for PI 3-kinase and other signaling pathways either impinge on common downstream targets, or utilize parallel pathways to fulfill the same functions.

2. Materials and methods

2.1. Materials

Histopaque 1077 was from Sigma Diagnostics (St. Louis, MO). Aluminum-backed Si 60 TLC plates were from E. Merck (Darmstadt, Germany). WM and LY-294002 were purchased from Sigma Chemical Co. and Calbiochem (La Jolla, CA), respectively. The enhanced chemiluminescence (ECL) system was from Amersham Life Sciences (Montreal, Canada). [γ-^{32}P]ATP (6000 Ci/mmol) was from Dupont-NEN Research Products (Boston, MA). [^{32}P]Orthophosphate (carrier-free) was from ICN Pharmaceuticals (Costa Mesa, CA). PI was purchased from Avanti Polar Lipids (Alabaster, AL). PI-4-P and PI-4,5-P_2 were from Boehringer Mannheim Canada (Laval, Quebec). Anti-PI 3-kinase (p85α polyclonal) and anti-phosphotyrosine (4G10 monoclonal) were from Upstate Biotechnology (Lake Placid, NY). Anti-PI 3-kinase (p110 β polyclonal) was from Santa Cruz Biotechnology (Santa Cruz, CA). PAC-1 antibody was purchased from Becton–Dickinson.

2.2. Platelet isolation

Rabbit platelets were isolated essentially according to the method of Pinckard et al. [15]. Whole blood from New Zealand white rabbits was expressed directly into anticoagulant (25 g trisodium citrate dihydrate, 14 g citric acid and 20 g glucose per liter) at a ratio of 9:1 and used immediately. Anticoagulated blood (30 ml) was diluted with 10 ml Tyrode's buffer pH 6.5 containing 0.1 mM EGTA and centrifuged for 12 min at $190\times g$ at room temperature. The platelet-rich plasma (8 ml) was withdrawn and underlayered with 2 ml Histopaque 1077 solution. Platelets, which formed a band at the Histopaque/plasma interface after centrifugation, were diluted with Tyrode's buffer+EGTA and the Histopaque separation repeated. The platelets were then washed three times and counted in an automated Coulter counter. For experiments, platelets were gently resuspended in Tyrode's buffer pH 7.2 containing 1 mM Ca^{2+}.

Human platelets were washed and isolated according to a previously described method [16]. Apyrase, heparin and prostaglandin I_2 were included in all steps after the initial centrifugation to inhibit premature activation.

2.3. Platelet aggregation and GPIIbIIIa activation

Platelets were resuspended to $2.5–5.0\times10^8$/ml in Tyrode's solution+1 mM Ca^{2+}. Aggregation was determined by the turbidimetric method in an aggreg-

ometer. The platelet suspension (0.5 ml) was transferred to a siliconized glass cuvette containing a small magnetic stir bar. The aggregating agent was added in a small volume (up to 50 μl) and light transmission at 609 nm was recorded continuously with a potentiometric pen recorder. Test compounds were preincubated for 10 min at 37°C with the platelet suspension. When the response to TRAP was being tested, 500 μg/ml fibrinogen was added.

For experiments measuring GPIIbIIIa activation, human platelets were incubated in the presence or absence of LY-294002, then diluted into buffer containing a final concentration of 0.5μg/ml PAC-1 antibody, along with agonists or blocking peptides or antibody. After mixing, platelets were incubated in the dark for 5 min and then PAC-1 binding was analyzed by flow cytometry.

2.4. Detection and quantitation of intracellular PIP_3 and PI-3,4-P_2

Platelets were labeled with ^{32}P by resuspending in Tyrode's–Hepes buffer, pH 7.0, to 5×10^9 per ml and addition of 0.75 mCi/ml $H_3[^{32}P]O_4$ for 90 min at 37°C. Excess label was removed by centrifugation, platelets were washed twice at room temperature with fresh medium, then suspended at 3×10^9/ml in Tyrode's–Hepes buffer+1 mM Ca^{2+}. Platelet suspensions (0.5 ml) were stimulated with agonist at 37°C in an aggregometer and reactions terminated by adding 50 μl concentrated HCl (1 N final) followed by chilling in an ice-bath.

Total platelet phospholipids were extracted by a modification of a previously described method [17]. Chilled platelet suspensions were transferred to 15-ml polypropylene tubes, then vortexed for 20 s with 1.88 ml 2:1 MeOH/$CHCl_3$ (v/v) and allowed to stand at room temperature for 20 min. Phases were separated by vortexing the mixture with 0.63 ml H_2O then 0.63 ml $CHCl_3$ followed by centrifugation. The lower phase was recovered, the upper phase re-extracted and the organic layers combined. Two washes of the organic layer with 0.5 ml 1:0.9 MeOH/0.1 M EDTA (v/v) were performed. The extract was transferred to glass tubes and dried under nitrogen.

For separation of PIP_3, the lipid film was dissolved in 100 μl 95:5 $CHCl_3$/MeOH and applied to the origin of a 20×20 cm oxalate-treated silica-gel TLC plate. The plate was developed to the top in the following solvent system; $CHCl_3$/acetone/MeOH/acetic acid/H_2O (80:30:26:24:14, v/v/v/v/v) [18], then dried and exposed to autoradiography film. Alternatively, the radioactivity was quantitated using a Molecular Dynamics Phosphorimager. The spot corresponding to ^{32}P-labeled PIP_3 in stimulated platelets was located by co-migration with authentic standard produced by in-vitro phosphorylation of PI-4,5-P_2 by PI 3-kinase. The PIP_3 spots were excised from the TLC plate and quantified by scintillation counting. Values were routinely normalized to total ^{32}P dpm in the extract.

PI-3,4-P_2 was measured by de-acylation of the PIP_2 zone (PI-3,4-P_2 and PI-4,5-P_2) on the TLC plate, followed by HPLC separation of the glycerophosphoinositides, as described previously [17]. For resolution of glycero-PI-3,4-P_2 (gPI-3,4-P_2) from gPI-4,5-P_2, a shallow gradient was employed. The injection was followed by a 10-min H_2O wash then a 60-min linear gradient to 0.25 M ammonium phosphate (pH 3.8), followed by a 50-min linear gradient to 1.0 M ammonium phosphate (pH 3.8). Under these conditions gPI-3,4-P_2 and gPI-4,5-P_2 eluted at 45.5 min and 48 min, respectively. ^{32}P-gPI-4,5-P_2 and ^{32}P- gPI-3,4-P_2 were identified by co-elution with deacylated lipid standards.

2.5. In vitro determination of phosphatidylinositol 3-kinase activity

Platelets were solubilized and PI 3-kinase activity was determined in immunoprecipitates essentially as described previously [19]. Briefly, platelets in Tyrode's–Hepes buffer, pH 7.2, containing 1 mM Ca^{2+} were solubilized with an equal volume of ice-cold 2×lysis buffer (2% Triton X-100, 20 mM Tris–HCl (pH 8), 137 mM NaCl, 20% glycerol, 4 mM EDTA, 2 mM Na_3VO_4, 2 mM Na_3MoO_4, 2 μM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 2 μg/ml aprotinin, 20 mM NaF, 2 μM pepstatin, 20 μg/ml soybean trypsin inhibitor). Detergent-insoluble materials were removed by centrifugation for 15 min at 14 000 rpm at 4°C. Platelet lysates were immunoprecipitated with a monoclonal anti-phosphotyrosine antibody (4G10), or rabbit antisera to the p85α or p110β subunits of PI 3-kinase, washed twice with lysis buffer and three times with buffer

consisting of 10 mM Tris–HCl, pH 7.4. Ten μg sonicated PI was mixed with the beads and kept for 10 min on ice. The kinase reaction was initiated by adding 40 μl of kinase buffer (50 μM ATP, 30 mM Hepes (pH 7.4), 30 mM $MgCl_2$, 200 μM adenosine) containing 10 μCi of [γ-^{32}P]ATP. After 15 min at room temperature, 0.1 ml of 1 N HCl and 0.2 ml of chloroform/methanol (1:1, v/v) were added to stop the reaction. The samples were vortexed then the lower organic layer (70 μl) transferred to new tubes and kept at −20°C until analysis.

^{32}P-labeled PI-3-P was separated from residual [γ-^{32}P]ATP (retained at the origin) by chromatography on oxalate-treated TLC plates using a solvent system of chloroform/methanol/water/28% ammonium hydroxide (90:70:15:5, v/v/v/v). TLC plates were exposed to X-ray film at −80°C then radioactivity incorporated into PI-3-P (PI 3-kinase activity) was measured by excising the spot from the plate followed by liquid scintillation counting.

3. Results

3.1. PIP_3 and PI-3,4-P_2 are elevated in PAF-stimulated platelets

Rabbit platelets were labeled for 90 min with [^{32}P]orthophosphate then incubated for various times with PAF under stirring conditions in an aggregometer, after which lipids were separated by TLC. The time course of appearance of PIP_3 was found to correlate closely with the induction of aggregation by PAF, with a significant elevation above basal levels observed within 30 s and a greater than 2-fold increase observed by 1 min (Fig. 1). PI-3,4-P_2 levels in the same samples were analyzed following elution of PI-bisphosphates from the TLC plates. The separation of deacylated PI-3,4-P_2 from deacylated PI-4,5-P_2 was achieved by HPLC separation of the corresponding glycero-phosphoinositides (Fig. 2). From the results of a typical experiment using the average of duplicate samples, PI-3,4-P_2 was was only slightly elevated above baseline counts after 5 s PAF treatment (210 cpm in control, 230 cpm after 5 s treatment). A larger increase in PI-3,4-P_2 was observed after 1 min, with 1160 cpm in the PI-3,4-P_2 peak, and this dropped to 710 cpm by 10 min. We have also

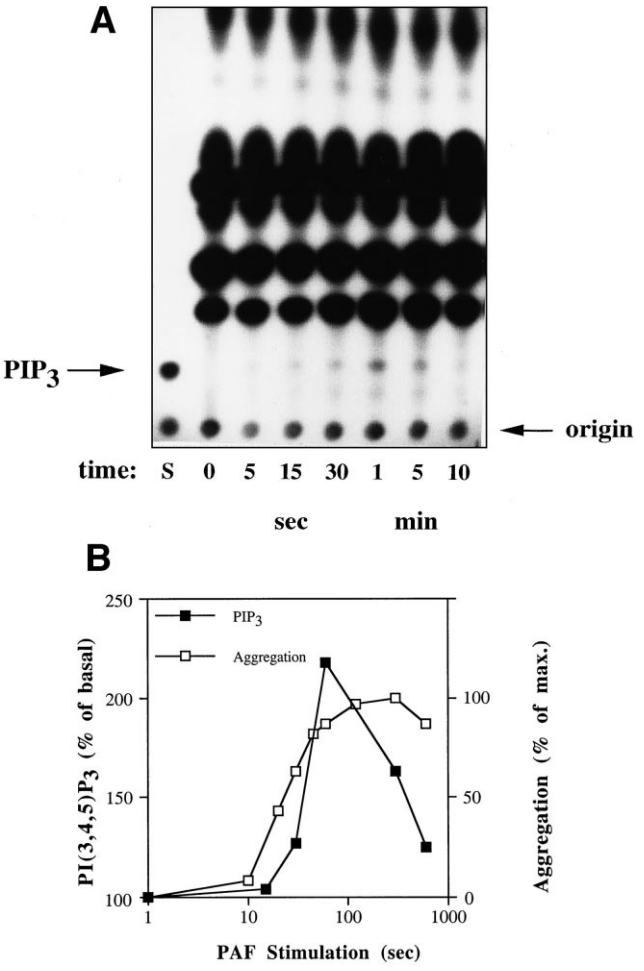

Fig. 1. PIP_3 elevation correlates with aggregation in PAF-stimulated rabbit platelets. ^{32}P-Labeled platelets were stimulated for various times with 100 nM PAF in an aggregometer then total phospholipid extracts separated by chromatography on oxalate-treated silica gel TLC plates. (A) Autoradiogram of TLC plate after 2 h exposure. PIP_3 in platelet samples is located by co-elution with authentic PIP_3 synthesized enzymatically. (B) The PIP_3 spot was excised and ^{32}P quantitated by scintillation counting. PIP_3 levels in PAF-stimulated samples are expressed as percentage of PIP_3 counts in unstimulated platelets (basal PIP_3 = 6000 cpm). Progress of platelet aggregation in samples subsequently extracted for PIP_3 measurements is plotted on the same graph. Data are in triplicate from a single experiment representative of several. The data from the 5 s time point was not plotted as it was not significantly different from controls. The maximum in PIP_3 varied between 30 s and 1 min for different experiments, consistent with the kinetics of aggregation.

found $PIP_3$to be elevated in thrombin-stimulated rabbit platelets with a time course that also closely correlates with aggregation (data not shown). These results suggest that the appearance of the products of

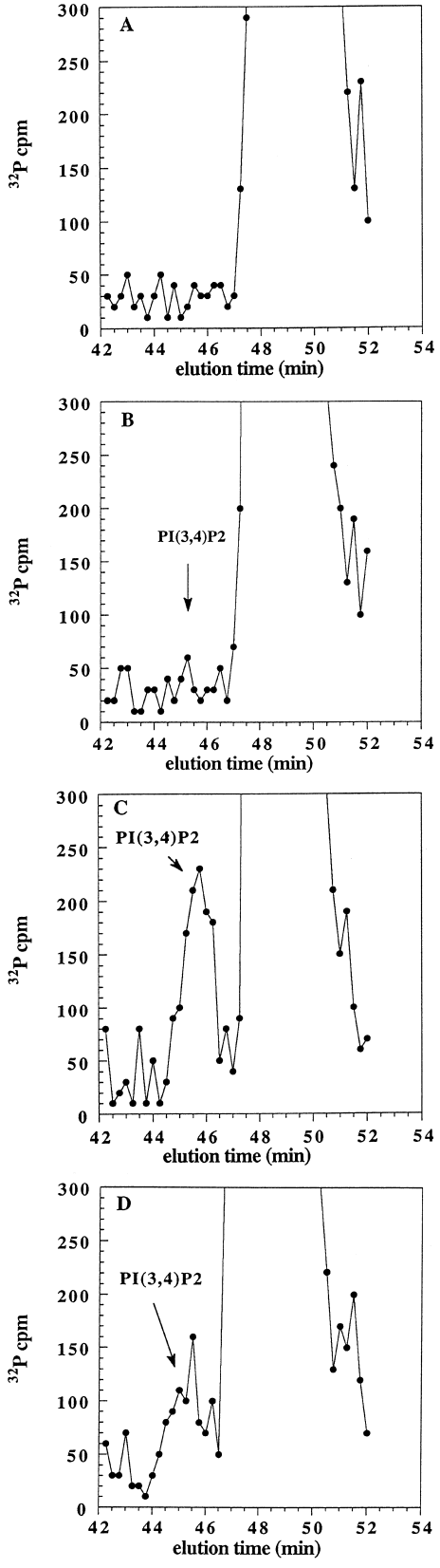

Fig. 2. PAF treatment of rabbit platelets induces formation of PI-3,4-P_2. De-acylated PI-bisphosphates isolated after TLC prepurification of lipid extracts from ^{32}P-labeled PAF-stimulated platelets were separated by strong anion exchange HPLC. Shown are representative tracings from four separate sample injections (unstimulated (A), 5 s (B), 1 min (C) and 10 min (D) PAF stimulation) after column fractions were counted in a scintillation counter. Arrow indicates the elution position of gPI-3,4-P_2. gPI-4,5-P_2 is the large peak eluting at 49 min.
←

activated PI 3-kinase in platelets may play a role in regulation of downstream events such as aggregation.

3.2. *Effect of PI 3-kinase inhibitors on PI 3-kinase activity and PAF-induced platelet aggregation*

Two potent and selective inhibitors of PI 3-kinase, WM and LY-294002, were tested on rabbit platelets for their ability to block the PAF-induced increase in PIP_3. WM inhibited PIP_3 formation with an IC_{50} between 10 nM and 40 nM. Similarly, LY-294002 half-maximally inhibited PIP_3 at 2–5 μM (data not shown). At concentrations of 100 nM WM or 50 μM LY-294002, complete inhibition of PI 3-kinase activity was observed, regardless of the concentration of agonist used. WM and LY-294002 also blocked the thrombin-induced increase in PIP_3 in rabbit platelets with a similar potency (data not shown). The IC_{50}s we found are very consistent with the ability of these two inhibitors to block PI 3-kinase activation in platelets stimulated with other agonists and in other cell types [12,20–23].

The ability of WM or LY-294002 to inhibit platelet aggregation was tested to determine whether PI 3-kinase activity is important for PAF-induced platelet aggregation (Fig. 3). An effect of the inhibitors on aggregation was found to be very dependent upon the concentration of PAF used, or more precisely, upon the activation state of the platelets. When rabbit platelets were stimulated with concentrations of PAF greater than 0.1 nM, they were quite resistant to the effects of WM or LY-294002. When 1 nM PAF was used, no inhibition of platelet aggregation was detected at concentrations of WM up to 1 μM (Fig. 3A), a concentration at which WM is known to inhibit other enzymes, in addition to PI 3-kinase [24,25]. Similarly, the highest concentration of LY-

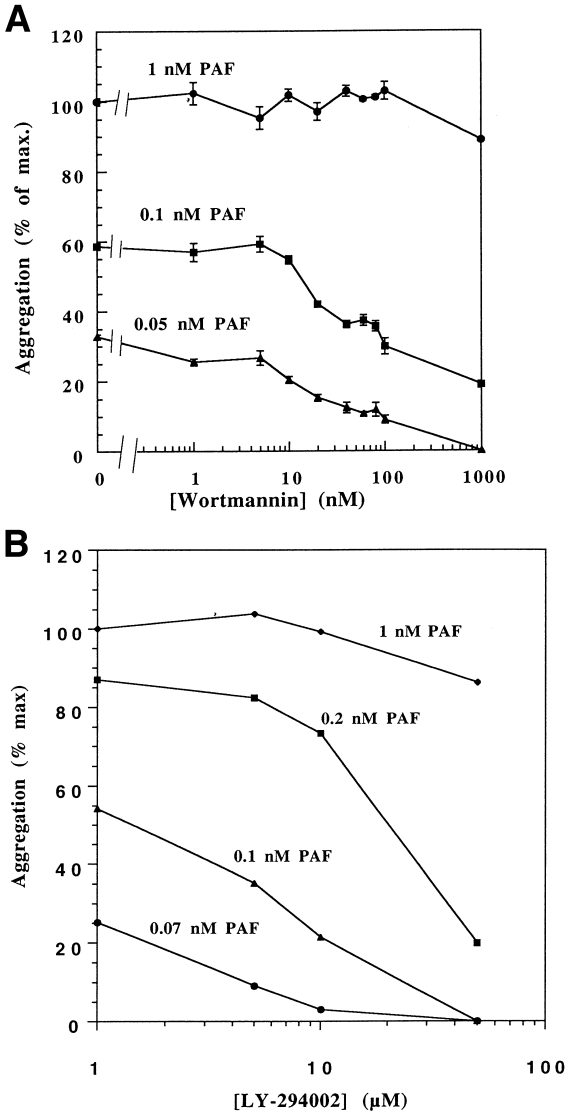

Fig. 3. Inhibition of aggregation by WM and LY-294002 depends on the activation state of the platelets. Rabbit platelets were preincubated with varying concentrations of WM (A) or LY-294002 (B) then stimulated for 1 min with the noted concentrations of PAF, and aggregation recorded in an aggregometer. PAF levels were chosen that induced reversible or irreversible aggregation in the absence of inhibitors and normalized to the level of aggregation observed at 1 nM PAF as 100%. Values are mean ± standard error of triplicate determinations from a single representative experiment which was repeated at least three times. DMSO concentrations did not exceed 0.2%.

294002 tested, 50 μM, which is a concentration that completely inhibits PI 3-kinase activity [26], gave only a slight inhibition of aggregation in response to 1 nM PAF (Fig. 3B). Below certain concentrations of PAF (usually 0.1 nM or less, depending upon the particular platelet preparation) platelet aggregation is reversible and exhibits only a primary phase (data not shown). If one examines platelets stimulated with 0.1 nM PAF, WM was able to inhibit aggregation with an IC_{50} less than 100 nM, and when platelets were stimulated with 0.05 nM PAF, the effective IC_{50} was even lower, showing that at these lower agonist concentrations, the effect of WM was observed at concentrations that correlated very closely with its inhibitory effect on PI 3-kinase activity. Similarly, LY-294002 inhibited aggregation in response to 0.1 nM PAF or lower with an IC_{50} of less than 10 μM (Fig. 3B).

3.3. *Effect of PI 3-kinase inhibitors on thrombin- or TRAP-induced platelet aggregation*

The effect of PI 3-kinase inhibitors on thrombin-induced aggregation in human platelets was also tested and similar results were obtained (Fig. 4). In this figure, aggregometer tracings are shown to demonstrate that with 1 U/ml thrombin, there is no effect of PI 3-kinase inhibitors, and no evidence of rever-

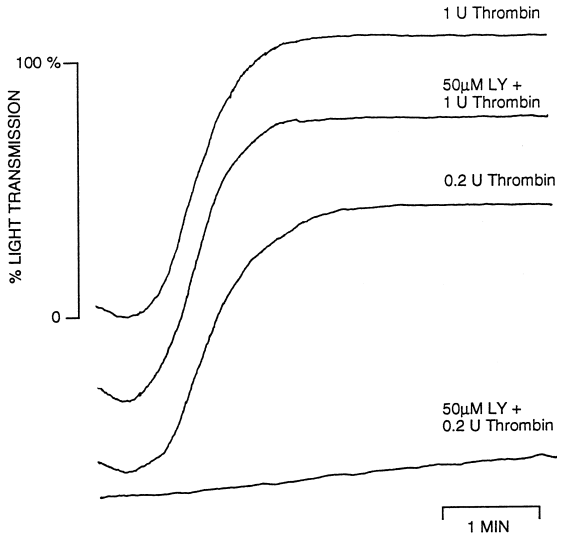

Fig. 4. Effect of PI 3-kinase inhibition on thrombin-induced aggregation. Human platelets were stimulated with either 0.2 or 1 U/ml thrombin in the presence or absence of LY-294002 as indicated. A representative set of tracings is shown, but similar results have been obtained in at least 10 independent experiments. In some cases, the extent of aggregation using 0.2 U/ml thrombin did not reach the same maximal level, depending on the individual donor, but the effect of the PI 3-kinase inhibitor was similar.

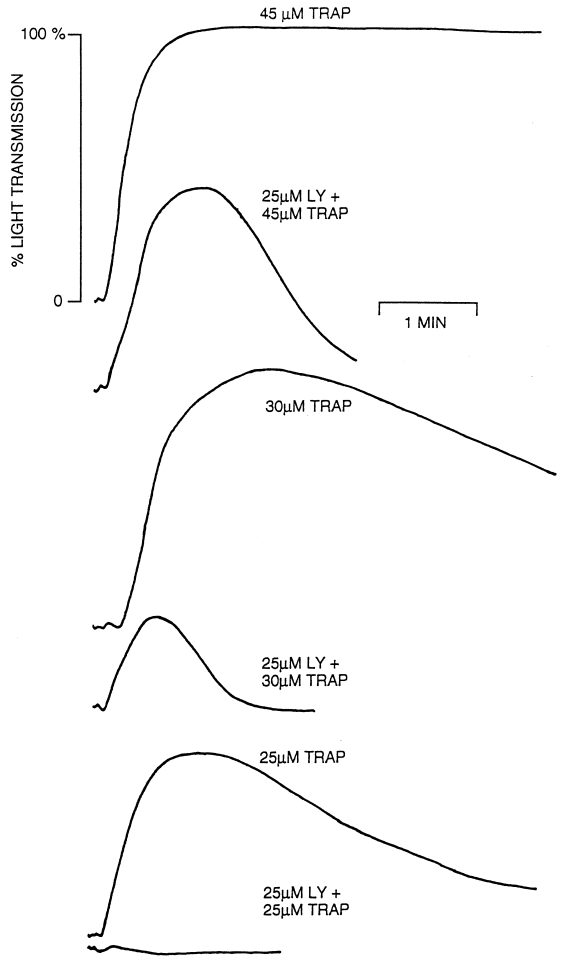

Fig. 5. TRAP-induced aggregation inhibited by LY-294002. Human platelets were stimulated with 25 μM, 30 μM, or 45 μM TRAP, the latter concentration giving maximal, irreversible aggregation. Preincubation with 25 μM LY-294002 caused reversible aggregation in response to 45 μM TRAP, caused greater inhibition in platelets stimulated with 30 μM TRAP, and almost completely inhibited aggregation induced by 25 μM TRAP. Average aggregometer readings are indicated, taken from a representative experiment.

sible aggregation. It should be stressed that the PI 3-kinase inhibitors, at optimal concentrations used in these experiments, are able to completely inhibit PI 3-kinase activated in response to 1 U/ml thrombin. When 0.2 U/ml thrombin was used, aggregation was almost to the same extent, and irreversible aggregation was also achieved. However, preincubation with 50 μM LY-294002 almost completely inhibited aggregation induced by 0.2 U/ml thrombin. Human platelets were also stimulated with various concentrations of TRAP to induce aggregation (Fig. 5). At the highest concentration of TRAP (45 μM), preincubation with LY-294002 at a concentration known to effectively block PI 3-kinase activity, caused reversible aggregation, with little effect on the initial phase of platelet aggregation, similar to results reported by Kovacsovics et al. [12]. However, at an intermediate concentration of TRAP, there was a significant inhibition of the early phase of aggregation, and at the lowest concentration of TRAP, 25 μM, preincubation with LY-294002 caused complete inhibition of platelet aggregation. It is important to note that at the higher concentrations of thrombin or PAF that caused irreversible aggregation, the PI 3-kinase inhibitors did not block aggregation and did not reverse aggregation, in contrast to the results shown here with TRAP.

3.4. Role of PI 3-kinase in activation of GPIIbIIIa

The inside-out signaling leading to changes in the platelet integrin GPIIbIIIa were shown to involve PI 3-kinase, as determined by flow cytometric studies measuring binding of the PAC-1 antibody (Fig. 6). When human platelets were stimulated with 1 U/ml thrombin, the PI 3-kinase inhibitor again had no effect on PAC-1 binding; however, at a lower concentration of thrombin (0.2 U/ml), blocking PI 3-kinase completely inhibited activation of GPIIbIIIa. As controls, addition of RGDS or the blocking anti-

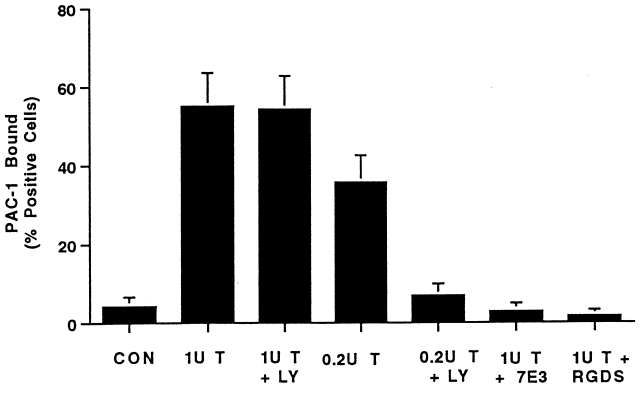

Fig. 6. Effect of PI 3-kinase inhibition on GPIIbIIIa activation. Human platelets were stimulated with 1 U/ml or 0.2 U/ml thrombin (1U T or 0.2U T) in the presence or absence of LY-294002 (LY) as indicated. Activation of GPIIbIIIa was measured using flow cytometry to measure binding of the PAC-1 antibody. Blocking of GPIIbIIIa using 7E3 or RGDS is presented as a control to show inhibition of PAC-1 binding.

body 7E3 were shown to inhibit binding of the PAC-1 antibody. Other controls (data not shown) showed that the peptide RGES, which does not bind to the integrin receptor, had no effect, and detection of GPIIbIIIa with another antibody that binds to all forms of the integrin showed that in all cases the same level of GPIIbIIIa expression could be detected.

3.5. Effect of PI 3-kinase inhibitors on secretion

The effects of the PI 3-kinase inhibitors on PAF induced platelet dense granule release were measured by quantitating the release into the medium of [^{3}H]hydroxytryptamine from preloaded rabbit platelets. Up to 30% of the release could be inhibited by concentrations of WM below 100 nM, or concentrations of LY-294002 below 10 μM, only when low concentrations of PAF were used, suggesting that a portion of the granules released depend upon PI 3-kinase, while the majority are not affected by blocking this activity. However, little or no inhibition of release was observed when a higher concentration of PAF was used. Time course studies showed that any inhibition of secretion by PI 3-kinase inhibitors was similar whether platelets were stimulated for 1 or 5 min with PAF, ruling out the possibility that the inhibitors were simply delaying the onset of release.

3.6. PI 3-kinase activity associated with tyrosine-phosphorylated proteins is increased in PAF-activated platelets

As we and others have shown, PAF or thrombin treatment of platelets results in an increase in tyrosine phosphorylation in platelets [3–5], mediated by as yet incompletely characterized tyrosine kinases. The mechanism of activation of PI 3-kinase in response to PAF was investigated by assaying for PI 3-kinase activity in anti-phosphotyrosine immunoprecipitates. Following activation by PAF, platelets were detergent-solubilized and extracts immunoprecipitated using 4G10 anti-phosphotyrosine antibody. PI 3-kinase activity was increased in the immunoprecipitates from PAF-activated platelets (Fig. 7). The amount of PI 3-kinase p85α subunit was increased in the anti-phosphotyrosine immunoprecipitates from PAF-treated platelets, correlating with the increase

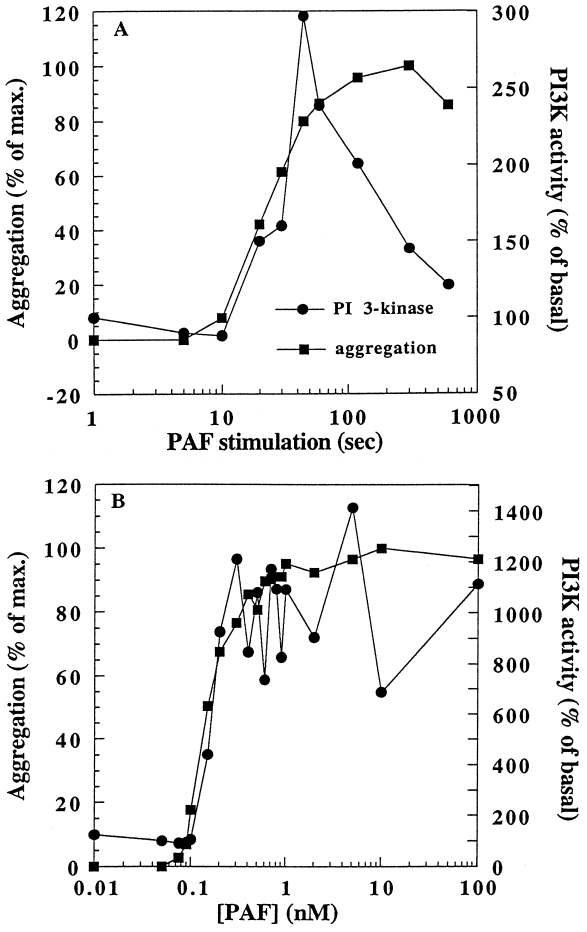

Fig. 7. Phosphotyrosine-associated PI 3-kinase activity is tightly linked to aggregation of PAF-stimulated rabbit platelets. Platelets were stimulated with 10 nM PAF for various times, solubilized in lysis buffer, then the supernatant remaining after centrifugation immunoprecipitated with monoclonal antibody to phosphotyrosine (4G10). PI 3-kinase activity was assayed in the immunoprecipitates using PI as a substrate. ^{32}P-labeled PI-3-P from TLC plates was excised and quantitated in a scintillation counter. Plots represent time-course (A) and concentration–response (B) curves for PI 3-kinase activity in anti-phosphotyrosine immunoprecipitates (●) co-plotted with aggregation (■) determined in the same samples. Data are averages of duplicate samples in a single experiment. Similar results were obtained in three independent experiments.

in PI 3-kinase activity, however there was no detectable increase in tyrosine phosphorylation of this subunit (data not shown). The latter result is consistent with the activation of PI 3-kinase in response to cytokines, in which no increase in tyrosine phosphorylated p85 was detected [17]. When the PI 3-kinase activity was quantitated and compared with platelet aggregation there was good correlation for both time

courses and concentration responses (Fig. 7A,B). This indicates a close temporal relationship between platelet aggregation, PI 3-kinase activation and association of PI 3-kinase with tyrosine-phosphorylated proteins. As expected, thrombin also stimulates an increase in PI 3-kinase activity associated with tyrosine-phosphorylated proteins in both rabbit and human platelets without any detectable increase in tyrosine phosphorylation of either subunit (data not shown).

4. Discussion

The activation of platelets by agonists such as PAF or thrombin leads to an extremely rapid series of signal transduction events that includes activation of tyrosine kinases, serine/threonine kinases, as well as lipid kinase activities, all within seconds to minutes of treatment [27]. The end result of these reactions is to cause platelet shape changes, secretion of granule contents and alterations in cell surface proteins, particularly the major platelet integrin, GPIIbIIIa, (or $\alpha_{IIb}\beta_3$) which is responsible for binding to fibrinogen. The engagement of GPIIbIIIa by dimeric fibrinogen and stirring leads to platelet aggregation and is also involved in the stimulation of 'late-phase' tyrosine kinase activity, subsequent to agonist stimulation [28]. In the past few years, some attention has been given to the role of PI 3-kinase activation in the events leading to platelet responses. Several studies have suggested that blocking PI 3-kinase activation in response to agonists such as phorbol ester, lysophosphatidic acid, serotonin/epinephrine and thrombin-receptor directed agonist peptide (TRAP) can at least partially block aggregation [12,23,29], although there is still little information about how the enzyme is activated in platelets and what specific signaling events or platelet responses are controlled in this pathway. One study showed that a role played by the D_3 phosphoinositides (products of PI 3-kinase activity) may be to control actin polymerization [30]. However, this was an event that was regulated following activation of the integrin receptor, GPIIbIIIa, and therefore cannot explain the role of PI 3-kinase in the inside-out signaling leading to activation of GPIIbIIIa. Recently, our group has described integrin-independent activation of the Pyk-2 tyrosine kinase in thrombin-treated platelets, and co-immunoprecipitation of Pyk-2 with PI 3-kinase [43].

In this study, we investigated the activation of PI 3-kinase in response to PAF treatment of rabbit platelets and found that this agonist is able to activate the enzyme. The most direct demonstration of PI 3-kinase activation in cells is obtained by determination of the amount of 3-phosphorylated phosphoinositides. We have shown that levels of PIP_3 and PIP_2 increase rapidly in response to PAF, with a time course that paralleled the aggregation of platelets (Fig. 1). The time-course we obtained for PAF-induced induction of PIP_3, reported here for the first time in platelets, is similar to that obtained when platelets are stimulated with thrombin [22,31,32]. However, thrombin stimulation usually results in a greater induction of PIP_3 (e.g., greater than 5-fold) than we observe for PAF [32]. The differences between PAF and thrombin with respect to PIP_3 induction are not unprecedented in that the TXA_2 mimetic, U46619 stimulates less PIP_3 than does thrombin and with different kinetics [30]. Also, another platelet agonist, lyso-phosphatidic acid, induces quite a different pattern of PIP_3 formation in platelets compared to thrombin or PAF [23]. PI-3,4-P_2 was also increased but with a slower and more prolonged time course than PIP_3 (Fig. 2), as has been observed by others [12,31–33]. It should be noted that we have shown that initial activation of PI 3-kinase activity by thrombin, as detected by immunoprecipitated enzyme activity, is not affected by addition of RGDS, showing that it is an early event, independent of platelet aggregation [43].

There was also an increase in PI 3-kinase activity associated with tyrosine phosphorylated proteins immunoprecipitated with an anti-phosphotyrosine antibody (Fig. 7), although there was no associated increase in tyrosine phosphorylation of the p85 subunit of PI 3-kinase (data not shown). We (data not shown) and others [17,34] have also found that thrombin causes an increase in PI 3-kinase activity associated with tyrosine-phosphorylated proteins. In agreement with our findings, strong platelet agonists generally do not induce tyrosine phosphorylation of the p85 subunit in platelets [35,36]. The only exception noted in the literature is one examining thrombopoietin stimulation [37]. This implies that for other platelet agonists, as well as for PAF, PI 3-kinase

associates with tyrosine-phosphorylated proteins, resulting in an increase in enzyme activity. Another group has shown that in a human B lymphoblastoid cell line, PAF stimulates an increase in PI 3-kinase activity associated with tyrosine-phosphorylated proteins, although this group did not quantitate 3-phosphoinositides directly [38]. Interestingly, they also detected PAF-induced tyrosine phosphorylation of the p85 subunit, suggesting that the mechanism of protein tyrosine kinase involvement in PAF-induced PI 3-kinase activation may be cell type-specific.

The role of PI 3-kinase activity in signal transduction events and downstream physiological responses can be studied by the use of selective inhibitors of this enzyme, WM and LY-294002. These inhibitors are structurally unrelated and inhibit PI 3-kinase by different mechanisms. WM forms a covalent linkage at the active site, while LY-294002 is a competitive inhibitor. As with all inhibitors, one must be cautious in claiming specific actions by any one compound. However, the availability of the two inhibitors that act by distinct mechanisms allows for a comparison of their actions on PI 3-kinase activity and on downstream targets, giving increased confidence in interpretation of the results. A recent study from our laboratory demonstrated the usefulness of such an approach when examining activation of PI 3-kinase and MAP kinase [39]. The in vivo IC_{50}s we determined for WM and LY-294002 inhibition of PAF induced PIP_3 formation (5–10 nM and 2–5 μM, respectively) agree with those found previously for both platelets and other cells [7,26,40]. When the effect of the inhibitors was tested on platelet aggregation, it was apparent that concentrations where the inhibitors were shown to be selectively blocking PI 3-kinase activity had little or no effect on aggregation induced by a maximal stimulus by PAF (Fig. 3) or thrombin (Fig. 4). When lower concentrations of agonists were used, particularly concentrations of the agonist that resulted in reversible platelet aggregation, then the inhibition by WM and LY-294002 correlated more closely with their ability to inhibit PI 3-kinase. These results suggest that PI 3-kinase plays a role in events leading to platelet aggregation, but it is a role that can be bypassed when higher concentrations of agonist are used.

A recent study showed that WM and LY-294002 could inhibit platelet aggregation induced by 25 μM TRAP with IC_{50}s of 50 nM and 25 μM, respectively [12], but the effect was primarily an inhibition of irreversible aggregation induced by the agonist. These authors concluded from further experiments that activation of PI 3-kinase is necessary for prolonged maintenance of the platelet glycoprotein GPIIbIIIa in an activated state and for irreversible aggregation, possibly through PI 3-kinase stimulation downstream of fibrinogen receptor engagement. However, when we examined the effect of PI 3-kinase inhibitors on TRAP-induced aggregation, we found that early stages of aggregation were also inhibited when lower concentrations of TRAP were used. It was clear that we could reproduce the effect of LY-294002 in causing reversible aggregation. However, this effect may be unique to the use of TRAP as an agonist, since irreversible aggregation induced by thrombin or PAF could not be reversed, but aggregation induced by lower concentrations of thrombin, which also gave irreversible aggregation, could be almost completely inhibited by LY-294002 (Fig. 4). When lower concentrations of TRAP were used, the PI 3-kinase inhibitor was able to completely block aggregation (Fig. 5). Therefore, from our results, we conclude that PI 3-kinase activity plays a very important role in the early events leading to platelet aggregation in response to low concentrations of platelet agonists. However, at higher agonist concentrations, PI 3-kinase activity can be bypassed, presumably by other signaling pathways. The later events leading to irreversible aggregation also appear to be partially dependent on PI 3-kinase, as in the case of TRAP stimulation or stimulation with lower agonist concentrations, but again this can be bypassed by strong platelet agonists. The activation of PI 3-kinase at low agonist concentrations appears to lead to the activation of GPIIbIIIa, as shown by flow cytometry studies in which the activated form of the receptor was detected using a specific antibody that binds to the activated GPIIbIIIa. Clearly, the ability of higher concentrations of thrombin to activate GPIIbIIIa in the absence of PI 3-kinase activity supports the other studies, suggesting that the higher agonist concentrations can activate pathways that bypass the need for PI 3-kinase.

One working hypothesis is that the activity of a downstream enzyme regulated by PI 3-kinase is necessary for platelet aggregation. A candidate for such

an enzyme might be one of the PKC family of kinases that are potentially regulated by phosphorylation pathways dependent upon PI 3-kinase, as has been shown recently [41]. For PKCδ, the activation has been shown to involve phosphorylation of the enzyme in the activation loop by the PIP_3-dependent kinase, PDK1 [41]. Clearly, this could be happening in platelets since we have previously demonstrated a potential modulation of PKCδ in platelets dependent upon PI 3-kinase activation [42]. However, it is possible that the PKC enzyme(s) may be regulated by more than one phosphorylation pathway, and thus the need for PI 3-kinase can be bypassed by alternate activation pathways. These alternate pathways could activate the same downstream protein kinase independently of PI 3-kinase, or a different protein kinase that can fulfill the same function. Our results are also consistent with the possibility that PI 3-kinase is playing a role in maintaining the fibrinogen receptor in an activated state. In the case of the agonist TRAP, there may be a greater need for PI 3-kinase to maintain the fibrinogen receptor in its activated state compared to other stronger agonists, which at higher concentrations can utilize PI 3-kinase-independent pathways. One might even speculate that effects via the different protease activated receptors, that are differentially activated by TRAP and thrombin, could provide a possible explanation for the differences. Finally, we cannot rule out the possibility that the inhibition of PI 3-kinase can have an effect by inhibition of platelet granule release. As mentioned, a partial inhibition of secretion was observed following incubation with PI 3-kinase inhibitors, which could possibly be affecting a subset of the granules, and these could be releasing agonists (e.g., ADP) that contribute to aggregation.

The role of the gamma isoform of PI 3-kinase (p110γ) in platelets was not investigated in this study, but its potential contribution to the formation of 3-phosphorylated inositol phospholipids cannot be overlooked. Detailed studies with this isoform will have to await the availability of better antibodies. However, it is clear from the PI 3-kinase inhibitor studies that the production of PI 3-kinase products was being completely inhibited at appropriate concentrations. Therefore, if p110γ was playing an important role, it too was being inhibited by the PI 3-kinase inhibitors and therefore our arguments regarding the role of PI 3-kinase can be generalized to all of the isoforms.

In conclusion, we have shown that PI 3-kinase is activated in PAF- or thrombin-stimulated platelets and plays an important role in the aggregation response at lower activation states, or in response to lower concentrations of agonist, which could still be physiologically relevant. PI 3-kinase signaling may be of less importance at higher activation states due to contributions from other signaling pathways that may impinge on a common downstream event. A key focus of future studies will be to examine the activity of kinases dependent upon PI 3-kinase activity, and determine how they are regulated at various agonist concentrations, in the presence or absence of PI 3-kinase inhibitors.

Acknowledgements

R.W.L. was the recipient of a Heart and Stroke Foundation of Canada studentship. V.D. is the recipient of a Medical Research Council of Canada/B.C. Lung Association scholarship. This work was supported by a grant from the Heart and Stroke Foundation of Canada. We would like to thank David Fong, Michael Scheid and Dr. Michael Gold for technical assistance and helpful discussions. We thank Dr. B. Coller for generously providing 7E3 antibody for blocking GPIIbIIIa.

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